

Photosynthesis and Retention of Zooxanthellae and Zoochlorellae Within the Aeolid Nudibranch *Aeolidia papillosa*

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Abstract. Both zooxanthellae and zoochlorellae are found in the cerata of *Aeolidia papillosa* after it has ingested symbiotic *Anthopleura elegantissima* containing these algae. High rates of photosynthesis were found in algae present in the cerata and in algae isolated from nudibranch feces. For algal cells present in the cerata of nudibranchs collected in June 1991, carbon fixation by zooxanthellae (1.18 ± 0.36 pg C/cell/h) was significantly greater than carbon fixation by zoochlorellae (0.55 ± 0.32 pg C/cell/h). Algal densities within the cerata of laboratory fed nudibranchs were significantly greater for zoochlorellae (175 ± 82 cells/ μ g protein, light treatment; 131 ± 106 cells/ μ g protein, dark treatment) than for zooxanthellae (38 ± 18 cells/ μ g protein, light; 53 ± 30 cells/ μ g protein, dark). Ceratal densities of zooxanthellae (16 ± 8 cells/ μ g protein) in the field during January 1992 were low in comparison to ceratal densities in the laboratory—several of the nudibranchs in the field lacked any symbiotic algae, and zoochlorellae were always absent. Nudibranch algal densities were not stable and dropped rapidly if the nudibranchs were starved. Both zoochlorella and zooxanthella densities dropped to 0 cells/ μ g protein within 11 days of starvation. While these results show that the relationship between *A. papillosa* and the two algae is not a stable symbiosis, the photosynthetic activity of the algae in the cerata suggests that the nudibranch and/or the algae may benefit from the association while it lasts.

Introduction

Several aeolid nudibranchs, as well as other nudibranchs with cerata, contain zooxanthellae of the genus

Symbiodinium (Rudman, 1981a, b, 1982; Kempf, 1984, 1991). Each cerata contains a diverticulum of the digestive gland within which the algal symbionts are both extra- and intracellularly located (Rudman, 1982; Kempf, 1984, 1991). Many of these nudibranchs obtain their algae through ingestion of marine cnidarians which are symbiotic with zooxanthellae. Zooxanthellae in the cnidarian host fix carbon through photosynthesis, and then translocate much of this carbon to the animal's tissue (e.g., Trench, 1979). The carbon available for translocation may represent as much as 95% of the amount fixed (Muscatine *et al.*, 1984), and is used by the host for respiration, growth, and reproduction (Kevin and Hudson, 1979; Davies, 1984; Rinkevich, 1989).

The ability of zooxanthellae to fix and translocate carbon in nudibranchs, as well as the benefits of such an association to the nudibranchs, have been described for several relationships. Crossland and Kempf (1985) reported that zooxanthellae in the tropical nudibranch *Melibe pilosa* fixed large amounts of carbon (5.85 mg C/mg chlorophyll *a*/h), and that fixed carbon was translocated to the nudibranch for growth and reproduction. Kempf (1990) reported that the aeolid nudibranch *Berghia verrucicornis* produced 1.7 times more eggs when in a symbiotic relationship with zooxanthellae than when algae-free. At high densities, zooxanthellae in the temperate nudibranch *Pteraeolidia ianthina* can supply carbon well in excess of the nudibranch's respiratory demand during the spring and summer (Høegh-Guldberg and Hinde, 1986; Høegh-Guldberg *et al.*, 1986). With the exception of the nudibranch *Pteraeolidia ianthina*, only tropical species have been studied, and all the studies have focused on species with zooxanthellae symbionts.

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The temperate nudibranch *Aeolidia papillosa* is found within the intertidal zone of the northeastern Pacific (Kozloff, 1983) where one of its preferred prey species is the symbiotic anemone *Anthopleura elegantissima* (Waters, 1973; Edmunds *et al.*, 1974; McDonald and Nybakken, 1978). *A. elegantissima* forms symbiotic relationships with both zooxanthellae and the unicellular green algae called zoochlorellae (Muscatine, 1971). Fixation and translocation of carbon by zooxanthellae in *A. elegantissima* is substantial (Trench 1971a, b), and the contribution of these anemones to intertidal gross primary production is equal to that of temperate intertidal seaweed populations on an areal basis (Fitt *et al.*, 1982). Zoochlorellae found in *A. elegantissima* and *A. xanthogrammica* are also photosynthetically active (Muscatine, 1971; O'Brien, 1980). While both zooxanthellae and zoochlorellae fix carbon in their host species, zooxanthellae translocate much more of their fixed carbon to their host than do zoochlorellae. Zooxanthellae translocate on the order of 50% of the carbon fixed while zoochlorellae translocate less than 5% (Muscatine, 1971; O'Brien, 1980).

High densities of zooxanthellae and zoochlorellae are found in the cerata of *A. papillosa* after it has been fed symbiotic anemones containing these algae (Kellett and Wiederspohn, pers. comm.). The following study considers the nature of the symbiotic relationship formed between *A. papillosa* and both zooxanthellae and zoochlorellae, particularly the photosynthetic activity of these algae and the stability of their populations within the nudibranchs' cerata.

Materials and Methods

Collection and maintenance of nudibranchs and anemones

Specimens of *A. papillosa* were collected from the San Juan Islands, WA in June 1991 and from the Port Orchard side of the Sinclair Inlet, WA in January 1992. All anemones used to feed the nudibranchs were collected from Skyline beach in Burrows Bay at Anacortes, WA. No nudibranchs were found on this beach. Individual nudibranchs were maintained in separate plastic mesh containers submerged in flow-through seawater tables at Shannon Point Marine Center (Anacortes, WA). Seawater tables were cleaned twice weekly. The temperature of the water during June 1991 ranged from 10.7°C to 13.0°C with a mean of 11.6°C. During January 1992 the temperature ranged from 7.5°C to 9.2°C with a mean of 8.4°C, and during February 1992 the temperature ranged from 8.2°C to 10.9°C with a mean of 9.1°C. The average salinity during the study was 29 ‰.

Specimens of *A. papillosa* collected in June 1991 were used to determine the productivity of symbiotic algae. Continuous light provided to nudibranchs by a bank of

two fluorescent lamps averaged 28 $\mu\text{mol photons/m}^2/\text{s}$ at the water's surface (LiCor cosine quantum sensor, 400–700 nm PAR). One group of nudibranchs was fed brown *A. elegantissima* containing zooxanthellae, another group was fed green *A. elegantissima* containing zoochlorellae, and the control group was fed white (algae-free) *A. elegantissima*. Personal experience has shown that brown anemones always contain at least 98% zooxanthellae (on a cell basis) and green anemones contain at least 98% zoochlorellae. This was confirmed during the experiment by periodic microscopic examinations of tentacle squashes from brown and green anemones.

Specimens of *A. papillosa* collected in January were separated into two groups of twelve to examine retention of zooxanthellae and zoochlorellae in the cerata. The initial algal complement of field nudibranchs was determined by sampling two cerata from each nudibranch within 24 h of collection. One group was maintained in continuous darkness, and the other group was maintained under 12 h light/12 h dark. During the light cycle, irradiance at the water's surface averaged 33 $\mu\text{mol photons/m}^2/\text{s}$. Each group in the light and the dark treatments was further separated into three treatments of 4 nudibranchs each. One group was fed brown anemones for 28 days, then had its diet switched to green anemones for 13 days, and was then starved. Another group was fed green anemones for 28 days and was then switched to a diet of brown anemones. The third group was fed brown anemones for 28 days and was then starved. Each fed nudibranch was given 5 anemones per week, provided individually on separate days. Fed nudibranchs had no more than two consecutive days without feeding.

Productivity of symbiotic algae

Algae within the cerata. To determine whether zooxanthellae and zoochlorellae remain photosynthetically active within the cerata of the nudibranchs, 3 cerata (one anterior, one middle, and one posterior) were removed from each nudibranch and incubated whole with ^{14}C in 20 ml glass scintillation vials. 2.0 ml of filtered seawater (FSW) and 0.1 $\mu\text{Ci } ^{14}\text{C}$ bicarbonate were added to each vial. The cerata were incubated at room temperature (20–24°C) at 249 $\mu\text{mol photons/m}^2/\text{s}$ for 50–120 min. Control vials for dark carbon fixation were also maintained for each ^{14}C experiment. Replicate vials of each treatment were wrapped with black electrical tape and incubated under the same conditions as light vials. The dark vials were used to correct for dark carbon fixation. To determine total activity (TA), 100 μl was subsampled from each vial and placed in a 7 ml plastic scintillation vial with 5 ml of Ecolume (ICN) scintillation fluid. Incubations were terminated by removing the cerata and washing them with several rinses of FSW. The cerata were then homogenized

in 1.5 ml of FSW using a 5-ml Wheaton tissue grinder. Two 500 μ l subsamples were taken from each homogenate solution and placed in separate plastic scintillation vials. All unfixed $^{14}\text{CO}_2$ was evolved from the homogenate subsamples by adding 300 μ l of 6N HCl and then placing the vials under a heat lamp in a fume hood for 1 h. The subsamples were neutralized with 300 μ l 6N NaOH prior to the addition of 5 ml scintillation fluid. The homogenate subsamples were counted along with the TA subsamples in a Packard TR 1900 scintillation counter using the automatic DPM mode. The remaining homogenate suspension was used for algal cell counts and then frozen for future protein analysis.

Algae isolated from nudibranch feces. Nudibranchs fed symbiotic *A. elegantissima* produced green or brown fecal pellets consisting mainly of intact symbiotic algae. Fresh fecal pellets were collected from nudibranchs and were suspended in FSW. Fecal algae were washed three times in FSW by centrifugation and resuspension. The final suspension was sequentially filtered through 73 μ m and 20 μ m Nitex screening to remove debris. After initial cell counts algal densities were adjusted to $4\text{--}6 \times 10^5$ cells/ml. The productivity of fecal algae was measured using a protocol similar to that described for the cerata with the following exceptions: 2.0 ml of either the green or the brown fecal algae suspension was placed in each 20 ml glass vial and the algal cells were allowed to incubate for 30 min at room temperature (20–24°C). Incubations were at an average irradiance of 249 μ mol photons/m²/s.

Algae freshly isolated from anemones. The productivity of algae isolated directly from *A. elegantissima* was also determined. The oral disk and tentacles of individual anemones were excised and homogenized using a tissue grinder. Algal cell suspensions were washed and filtered as described above. Final algal densities ranged from $2.5\text{--}6 \times 10^5$ cells/ml. Incubations were performed as above except that cells were allowed to incubate for up to 1 h at an average irradiance of 102 μ mol photons/m²/s.

Algal densities and replacement within the cerata

Algal population density within the cerata was measured twice each week by removing 2 cerata (one posterior and one anterior) from each experimental nudibranch during January and February 1992. The cerata were homogenized in 1.5 ml of cold FSW using a 2-ml Wheaton tissue grinder. Algal cell counts of the homogenate solutions were determined using a hemocytometer, and the remaining homogenate solutions were frozen for future protein analysis. Protein analysis was performed using the Lowry method (Lowry *et al.*, 1951) and bovine serum albumin (BSA) standards with the modification that the homogenates and standards were pre-treated at 30°C for 30 min in 0.1 N NaOH to solubilize the proteins. Cell

counts and protein content were used to determine cell densities within the cerata of nudibranchs fed green and brown *A. elegantissima*.

Statistical analyses

Comparison of photosynthetic rates. Photosynthesis data for algae in *A. papillosa* cerata, algae freshly isolated from *A. papillosa* feces, and freshly isolated from *A. elegantissima* were analyzed to determine if there was a significant difference in the rates of carbon fixation for zooxanthellae and zoochlorellae. Zooxanthellae rates of carbon fixation were compared to zoochlorellae carbon fixation rates using two-sample t-tests. Algae in cerata, algae from feces, and algae from anemones were all compared separately. Comparisons were also made of the photosynthetic rates of zooxanthellae and zoochlorellae between the different treatments.

Comparison of algal densities. Algal densities in *A. papillosa* cerata after 28 days of feeding the nudibranch either brown or green anemones were analyzed using two-sample t-tests to determine if there was a significant difference in the densities of zooxanthellae and zoochlorellae found in the nudibranchs both under light and dark conditions. When zooxanthellar densities were compared to zoochlorellar densities, the data were logarithmically transformed to correct for differences in variance between the algal types. The effect of light versus dark on zooxanthellar and zoochlorellar densities was also analyzed using two-sample t-tests.

Comparison of treatment effect on algal replacement. Repeated-measures analysis of variance (Potvin and Lechowicz, 1990) was used to analyze the effect of light versus dark on the replacement (after switching diets) and expulsion (during starvation) of algae in the cerata. Zooxanthellae data for the replacement of zoochlorellae with zooxanthellae and the expulsion of zooxanthellae were logarithmically transformed to correct for differences in variance between the light and dark treatments.

Results

Productivity of symbiotic algae

Both zooxanthellae and zoochlorellae remain photosynthetically active within the nudibranch cerata (Fig. 1), where the mean rate of carbon fixation by zooxanthellae is significantly greater ($P = 0.0216$) than that of zoochlorellae. Cerata used for determining the photosynthetic rate of zooxanthellae contained 99.9% zooxanthellae on a cell basis. Cerata used for determining photosynthetic rate of zoochlorellae contained 99.5% zoochlorellae.

Figure 1 also shows that algal symbionts isolated from nudibranch feces also had high photosynthetic rates. There is no significant difference ($P = 0.0781$) between the pho-

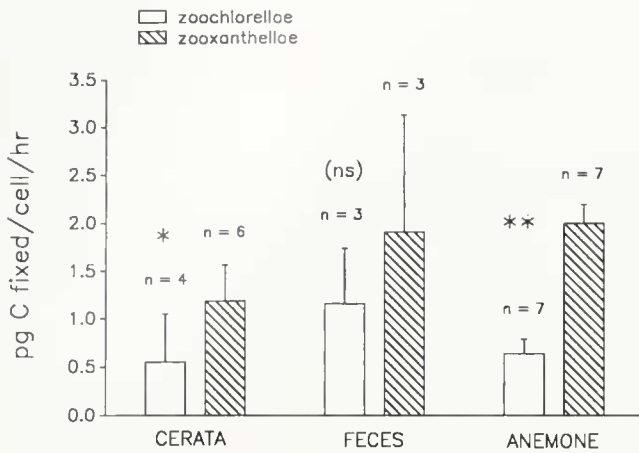


Figure 1. Mean rates of photosynthesis for algae incubated within whole cerata (*A. papillosa*) and for algae isolated from *A. papillosa* feces and from symbiotic anemones (*A. elegantissima*). Photosynthesis was determined in July 1991 at an average irradiance of 249 $\mu\text{mol}/\text{m}^2/\text{s}$ for cerata and fecal algae and in May 1991 at an average irradiance of 102 $\mu\text{mol}/\text{m}^2/\text{s}$ for anemone algae. Vertical lines represent 95% confidence intervals. Numbers above each bar represent treatment size. *, $P < 0.05$; **, $P < 0.0001$.

photosynthetic rate of zooxanthellae and that of zoochlorellae isolated from the nudibranch feces. But there is a significant difference ($P < 0.0001$) between the photosynthetic rate of zooxanthellae and the photosynthetic rate of zoochlorellae isolated from *A. elegantissima*.

In cross comparisons, photosynthesis by fecal zoochlorellae is significantly higher than photosynthesis of both zoochlorellae in the cerata ($P = 0.0369$) and zoochlorellae from the anemone ($P = 0.0033$). Photosynthetic rates are not significantly different between zoochlorellae in the cerata and zoochlorellae isolated from the anemone ($P = 0.5493$). For zooxanthellae, photosynthesis in the cerata is significantly lower than photosynthesis by both fecal zooxanthellae ($P = 0.0375$) and anemone zooxanthellae ($P = 0.0003$). The latter two are not significantly different from each other ($P = 0.6772$).

Algal densities within the cerata

Algal densities in freshly collected nudibranchs (field) during January 1992 averaged 16 zooxanthellae/ μg protein and ranged from 0 to 69 zooxanthellae/ μg protein. Only zooxanthellae were found within the cerata of the 24 nudibranchs collected from one beach on the Sinclair Inlet during a single low tide. No symbiotic algae were found in the cerata of nudibranchs collected in June 1991. These nudibranchs were collected from beaches where symbiotic anemones were not available as a food source. The algal densities in the cerata of field nudibranchs were low in comparison to those of nudibranchs regularly fed symbiotic anemones in the laboratory. These nudibranchs

ingested an average of 4.5 anemones each per week regardless of whether they were fed anemones with zooxanthellae or zoochlorellae.

Comparisons of algal densities between the light and dark treatments showed no effect from light on the densities of zooxanthellae or zoochlorellae in nudibranchs fed brown or green anemones, respectively. Zooxanthellae densities in nudibranchs maintained in the light (38 ± 18 cells/ μg protein) were not significantly different ($P = 0.3063$) from those in nudibranchs maintained in the dark (53 ± 30 cells/ μg protein). Zoochlorellae densities in the light treatment (175 ± 82 cells/ μg protein) were not significantly different ($P = 0.3339$) from those in the dark treatment (131 ± 106 cells/ μg protein).

Nudibranchs fed green anemones contained significantly higher algal densities than nudibranchs fed brown anemones ($P = 0.0004$ for nudibranchs maintained in the light; $P = 0.0152$ for nudibranchs maintained in the dark). After 28 days of feeding on one type of anemone, the cerata of nudibranchs fed brown anemones contained 99.9% zooxanthellae and the cerata of nudibranchs fed green anemones contained 99.0% zoochlorellae.

Replacement of algal populations in the cerata

Algal densities within the cerata do not remain constant over time and depend on the algal complement of the food source. When the nudibranchs were switched from a diet of anemones containing zoochlorellae to a diet of anemones containing zooxanthellae, the zoochlorellae within the cerata were completely replaced by zooxanthellae within 24 days (Fig. 2), after approximately 15 anemones containing zooxanthellae had been ingested per nudibranch. There is no significant difference between the light and dark treatments in either the loss of zoochlorellae ($P = 0.8338$) or the gain of zooxanthellae ($P = 0.0561$).

The replacement of zooxanthellae with zoochlorellae in the cerata required even less time. Upon switching the diet of nudibranchs from anemones containing zooxanthellae to anemones containing zoochlorellae, the zooxanthellae were replaced by zoochlorellae within 13 days (Fig. 3), after approximately 8 anemones with zoochlorellae had been ingested per nudibranch. Again, there is no significant difference between light and dark treatments in either the loss of zooxanthellae ($P = 0.7988$) or the gain of zoochlorellae ($P = 0.6664$).

The time it took for each type of alga to be completely expelled from the cerata upon starvation was even less than the algal replacement times. All algae disappeared from the cerata within 11 days when nudibranchs containing either zooxanthellae or zoochlorellae were starved (Figs. 3, 4). There is no significant difference between light and dark treatments in either the expulsion of zooxan-

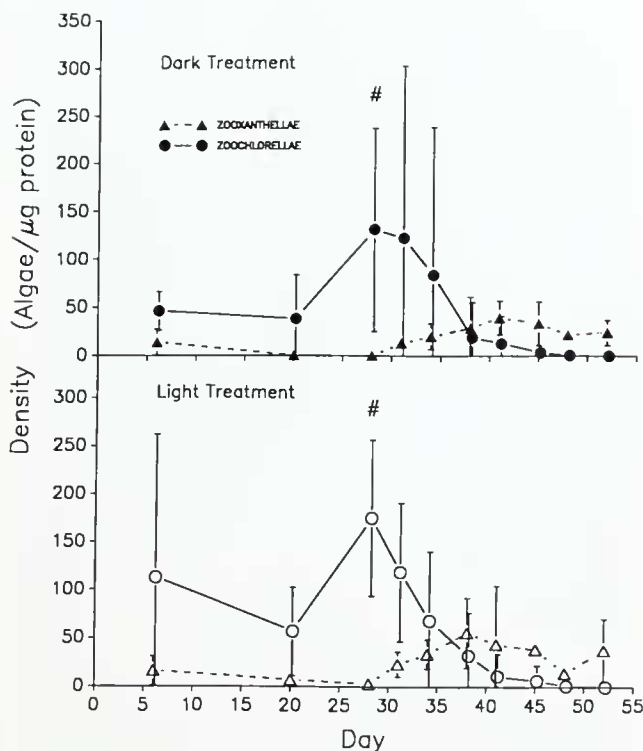


Figure 2. Mean algal densities in the cerata of nudibranchs initially fed anemones containing zoochlorellae, and then switched to anemones containing zooxanthellae on day 28 of the experiment. Closed symbols represent dark treatments and open symbols represent light treatments. Δ = zooxanthellae; \circ = zoochlorellae. Vertical lines represent 95% confidence intervals. The size of each treatment was 4 nudibranchs. # = day diet switched.

thellae ($P = 0.6674$) or the expulsion of zoochlorellae ($P = 0.1337$) during starvation.

Discussion

Since both zooxanthellae and zoochlorellae obtained by the ingestion of *Anthopleura elegantissima* remain photosynthetically active within the cerata (Fig. 1), it is likely that *Aeolidia papillosa* derives some benefit from these algae. Because of the higher density of zoochlorellae than zooxanthellae in the cerata, the actual amount of carbon fixed per ceras is not as different as the algal productivities would imply. The lack of any significant difference between the photosynthetic rates of fecal zooxanthellae and zoochlorellae may be due simply to the limited number of replicates. Although incubations were carried out at different irradiances ($249 \mu\text{mol photons/m}^2/\text{s}$ for ceratal and fecal algae, $102 \mu\text{mol photons/m}^2/\text{s}$ for anemone algae), and algae in cerata are likely to receive lower light during incubations than the isolated algae, both irradiances are well above the I_k value determined for zooxanthellae and zoochlorellae ($I_k = 50 \mu\text{mol photons/m}^2/\text{s}$)

in independent experiments also conducted during the summer (Aagaard and Muller-Parker, unpub.).

Zooxanthellae release much more of their photosynthate than do zoochlorellae within their primary hosts *A. elegantissima* and *A. xanthogrammica* (Muscatine, 1971; O'Brien, 1980). If this is also true within the nudibranch's cerata, there may be an energetic advantage to the selection of prey anemones containing zooxanthellae. *A. papillosa* does not appear to selectively retain one alga over the other since the expulsion of algae upon starvation was the same for both algae (Figs. 3, 4). The replacement of zoochlorellae with zooxanthellae took longer than the reverse. This may have been due in part to the size difference of the algae; the larger zooxanthellae may have restricted the exit of smaller zoochlorellae from the diverticula within the cerata.

Any benefits to the nudibranch of containing photosynthetically active algae would be most evident under light conditions. Therefore, if the nudibranch had the ability to control expulsion rates, algae should be retained longer under light than under dark conditions, especially

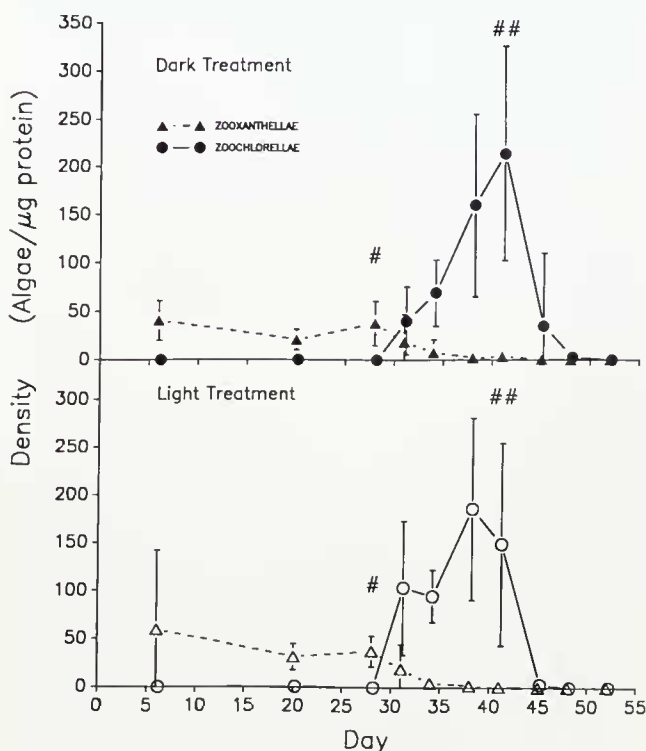


Figure 3. Mean algal densities in the cerata of nudibranchs initially fed anemones containing zooxanthellae, then switched to anemones containing zoochlorellae on day 28 of the experiment, and then starved after day 41 of the experiment. Closed symbols represent dark treatments and open symbols represent light treatments. Δ = zooxanthellae; \circ = zoochlorellae. Vertical lines represent 95% confidence intervals. The size of each treatment was 4 nudibranchs. # = day diet switched; ## = day began starving.

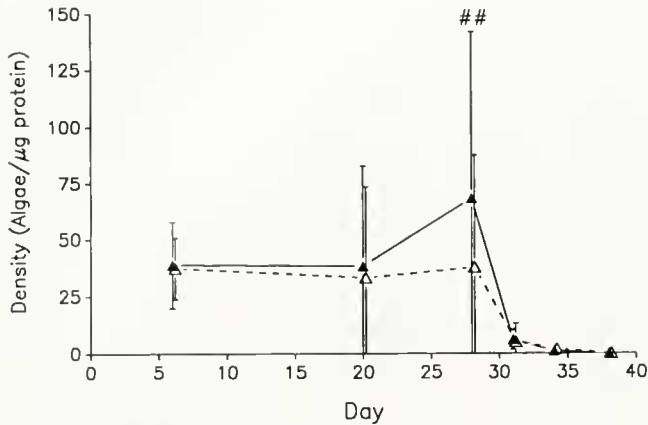


Figure 4. Mean zooxanthellae densities in the cerata of nudibranchs initially fed anemones containing zooxanthellae, and then starved after day 28 of the experiment. Closed symbols represent dark treatments and open symbols represent light treatments. Vertical lines represent 95% confidence intervals. The size of each treatment was 4 nudibranchs. ## = day began starving.

if the nudibranch is starved. The rapid expulsion of algae under both light and dark conditions suggests that *A. papillosa* has little control over the retention or expulsion of algae from its cerata, even when starved.

The large numbers of healthy algal cells present in fecal pellets and the photosynthetic rates of the fecal algae (Fig. 1) indicate that at least a portion of the algae consumed by the nudibranch pass unharmed through the digestive tract. Kempf (1984) found evidence of algal breakdown within the tissues of three tropical nudibranchs, but no evidence of active digestion in two additional species (Kempf 1984, 1991). Whether *A. papillosa* digests some of the ingested symbiotic algae is unknown, but at least a large number of the algae remain unaffected by passage through the nudibranch. Thus, the fecal material of *A. papillosa* may be important in the dispersal of algae and reinfection of temperate anemones as has been suggested for *Berghia major*, a tropical nudibranch that also feeds on symbiotic anemones (Muller Parker, 1984).

Another possibility is that the algae are heterotrophic in the nudibranch and thus represent a liability. Zooxanthellae isolated from the sea anemone *Aiptasia pulchella* are capable of heterotrophic growth under low light levels (Steen, 1987). The possibility of zoochlorellae being parasitic in *A. elegantissima* has been suggested by Muscatine (1971). Because of the generally low light levels in the Northeastern Pacific region, especially during winter months, algae within the cerata of the nudibranch may not be able to meet their carbon requirements photosynthetically. As such, it is possible that algae in *A. papillosa* are a benefit during the summer and a liability during the winter.

Kempf (1990, 1991) suggested that the nudibranch *Berghia verrucicornis* has a primitive mutualistic symbiosis with zooxanthellae based on the following observations. Relatively high concentrations of zooxanthellae are found in all *B. verrucicornis* from the field. The zooxanthellae (1) reside in peri-algal vacuoles within the nudibranch's digestive cells, (2) do not appear to be digested along with their primary host *Aiptasia pallida*, (3) remain photosynthetically active within the nudibranch, and (4) appear to benefit the nudibranch in its reproductive effort. Kempf terms the relationship primitive because the symbiosis is not permanent. The zooxanthellae are eventually exocytosed back into the gut and defecated in a healthy state when nudibranchs are starved in the laboratory.

The relationship between zooxanthellae, zoochlorellae, and *A. papillosa* may also be a primitive form of symbiosis, possibly corresponding to a Type IV association as described by Kempf (1991). Both algae are photosynthetically active within the nudibranch's cerata (Fig. 1). Rapid reduction in the density of each alga when it is no longer available in the nudibranch's food shows that frequent ingestion of symbiotic anemones is required to maintain the association. But *A. papillosa* does not appear to be obligately dependent on either alga at any period of the year. Several of the *A. papillosa* collected in the Sinclair Inlet lacked symbiotic algae in their cerata, and all of the *A. papillosa* collected in June 1991 on beaches where symbiotic *Anthopleura sp.* were not available, lacked symbiotic algae in their cerata. Ultrastructural investigations are needed to determine whether the algae are intracellular, and whether they reproduce while in the cerata. Translocation experiments to determine whether fixed carbon is utilized by the nudibranch for growth or reproduction will help to explain the nature of this relationship. Our work to date suggests that *A. papillosa* will be a good model system for comparing and contrasting the symbiotic relationships between zooxanthellae and zoochlorellae and their animal host.

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